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Accelerated Articles

Enhanced Electron Transfer Dissociation through Fixed Charge Derivatization of Cysteines

Lisa Vasicek and Jennifer S. Brodbelt*

Department of Chemistry and Biochemistry, The University of Texas at Austin, 1 University Station A5300, Austin, Texas 78712

Electron transfer dissociation (ETD) has proven to be a promising new ion activation method for proteomics applications due to its ability to generate c- and z-type fragment ions in comparison to the y- and b-type ions produced upon the more conventional collisional activation of peptides. However, low precursor charge states hinder the success of electron-based activation methods due to competition from nondissociative charge reduction and incomplete sequence coverage. In the present report, the reduction and alkylation of disulfide bonds prior to ETD analysis is evaluated by comparison of three derivatization reagents: iodoacetamide (IAM), *N*,*N*-dimethyl-2-chloro-ethylamine (DML), and (3-acrylamidopropyl)-trimethyl ammonium chloride (APTA). While both the DML and APTA modifications lead to an increase in the charge states of peptides, the APTA-peptides provided the most significant improvement in percent fragmentation and sequence coverage for all peptides upon ETD, including formation of diagnostic ions that allow characterization of both the C- and N-termini. In addition, the formation of product ions in multiple charge states upon ETD is minimized for the APTA-modified peptides.

There have been tremendous inroads in methodologies for the activation and dissociation of ions for proteomics applications in recent years. Although collision induced dissociation (CID) remains one of the most popular activation methods, largely due to a well-developed understanding of peptide fragmentation patterns,[1−3] other methods that address some of the more challenging sequencing problems have gained momentum. For example, because of its tunable and well-defined energy deposition, surface induced dissociation has proven to be very effective for peptide fragmentation, as well as more recently for examination of protein macromolecular complexes.[4] Photodissociation has also emerged as a promising alternative ion activation method for biological molecules, offering efficient energization of ions via a collision-free photoabsorption process.[5,6] Most recently, the development of electron-based dissociation methods, including electron capture dissociation (ECD)[7,8] and electron transfer dissociation (ETD),[9,10] has further expanded the suite of ion activation strategies used for proteomic applications.

The electron-based activation methods entail reactions of low energy electrons (ECD) or radical anions (ETD) with multiply charged peptides or proteins, leading to preferential cleavages of the $N-C_\alpha$ bonds along the backbone that result in the production of complementary c- and $z^\bullet$-type ions. The peptide length and amino acid composition have shown little influence on the fragmentation patterns of these dissociation methods in large scale studies,[11] although two more detailed targeted studies have reported some dependence.[12,13] ECD and ETD have proven to be particularly well-suited for the identification of

* To whom correspondence should be addressed. E-mail: jbrodbelt@mail.utexas.edu.

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post-translational modifications of proteins as these labile modifications are retained on the amino acid side chains during ion activation and dissociation and thus can be readily tracked, whereas they are often lost with other ion activation methods. Despite their tremendous promise, the electron-based dissociation methods exhibit a significant charge state dependence reflected by the fact that ions in lower charge states tend to undergo charge reduction without subsequent dissociation into informative c and $z^{\bullet}$ sequence ions.[8,14,15] The use of trypsin as a standard enzymatic reagent to cleave proteins prior to MS/MS analysis has in some ways exacerbated the tendency for charge reduction upon ECD or ETD because the large natural abundance of lysine and arginine residues in proteins results in the production of relatively short peptides upon tryptic digestion that yield lower charge states upon ESI.

Several methods have been aimed at alleviating this charge-state dependence of ETD and ECD, either utilizing auxiliary energization of ions to convert them to more meaningful sequence ions[14−20] or by increasing the charge states of the precursor ions prior to activation.[16−19] The former has been achieved by implementing supplemental collisional activation of the charge-reduced nondissociated precursor ions or by irradiating the precursor ions with photons to disrupt the noncovalent bonds formed between c- and $z^{\bullet}$-type product ion pairs.[14−20] Elevating the temperature of the ions in an ion cyclotron resonance (ICR) cell via blackbody heating provided sufficient energy to the precursor ions to destroy the noncovalent bonding and increase the formation of c- and $z^{\bullet}$-type fragment ions upon ECD.[20,21] In a process termed activated ion-ECD (AI-ECD) by McLafferty et al., simultaneous collisional activation and electron irradiation (i.e., ECD) of precursor ions also proved to be a successful approach for alleviating the extent of nondissociative charge reduction of precursor ions.[22,23] Both the Coon and McLuckey groups have employed supplemental collisional activation of charge-reduced products from electron transfer dissociation reactions, improving peptide sequence coverage.[11,24,25] More recently, the Coon group implemented photoirradiation during ETD of peptides in a linear ion trap mass spectrometer in a method analogous to AI-ECD described above.[26] This method significantly improved the sequence coverage of peptides having higher precursor m/z values (i.e., lower charge states).

Another approach to alleviating the charge state dependence of ETD efficiency is to produce precursor ions in higher charge states either through supercharging or derivatization techniques. The addition of nitrobenzyl alcohol to the ESI solutions promotes supercharging of ions, and subsequent ETD analysis of these highly charged precursor ions was enhanced through higher sequence coverage.[27] Another method was recently described for the analysis of peptides by derivatization of cysteine residues in peptides using *N*,*N*-dimethyl-2-chloro-ethylamine, which converts cysteines to highly basic dimethyl lysine (DML) moieties leading to higher net charge states upon ESI and greater peptide sequence coverage upon ETD.[28] Alternatively, the analyte molecules of interest can be derivatized by attachment of fixed charge sites to shift them to higher charge states prior to electron-based activation.[16−19] McLuckey et al. incorporated fixed charges via attachment of trimethylammonium moieties to primary amine groups in disulfide-linked peptides and demonstrated that the relative preference for backbone cleavage or disulfide bond cleavage upon ETD was influenced by the number of mobile protons versus fixed charges.[18] Other groups have demonstrated the benefits of fixed charge derivatization of peptides in conjunction with ECD. For example, Chamot-Rooke at al. showed that the addition of trimethyl-phenyl phosphonium cations to the N-termini of *O*-glycosylated and *O*-phosphorylated peptides increased the sequence coverage upon ECD.[16,17] O'Connor et al. reported the derivatization of lysine side-chains and/or N-termini via attachment of trimethylpyridinium and noted an increase in side-chain cleavages with a corresponding decrease in backbone cleavages upon ECD.[19]

In this study, we explore an alternative derivatization strategy based on the use of an S-alkylation reagent, (3-acrylamidopropyl)-trimethyl ammonium chloride (APTA), which introduces fixed charges to all free cysteine side-chains of peptides through a quaternary ammonium moiety, in conjunction with ETD analysis. The APTA reaction has been shown previously to improve the ionization efficiencies of peptides, as well as their water solubility, while also allowing the use of strong cation exchange chromatography for the separation of the derivatized peptides.[29,30] Our primary interest was exploiting the addition of fixed charge sites to enhance the use of electron transfer dissociation for the characterization of cysteine-containing peptides. In this study, APTA is used to derivatize cysteine-containing peptides, including those from a tryptic digest of bovine serum albumin (BSA), prior to characterization by ETD-MS.

EXPERIMENTAL SECTION

Materials and Reagents. All reagents were purchased from Sigma Aldrich (St. Louis, MO), and all peptides were

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obtained from BACHEM (King of Prussia, PA), including FQVVCG, HCKFWW, CDPGYIGSR, NRCSQGSCWN, somatostatin (AGCKNFFWKTFTSC), TGF-α (CHSGYVGVRC), and bactenecin (RLCRIVVIRVCR). Immobilized TPCK trypsin beads were purchased from Pierce Biotechnology (Rockford, IL). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ).

Mass Spectrometry. Samples were diluted to 10 μM in 49.5/49.5/1 water/methanol/acetic acid solution (v/v/v) and were directly infused at 3 μL/min into a Thermo Fisher LTQ XL (San Jose, CA) with ETD capabilities. All ETD experiments were conducted with fluoranthene as the radical anion electron-transfer reagent with a 100 ms reaction time. A Dionex UltiMate 3000 capillary high-performance liquid chromatograph (Sunnyvale, CA) was used for all separations. Samples with a final concentration of either 10 μM enzymatically digested protein or APTA-derivatized protein (1 μL injections) were introduced to a reversed-phase C_{18} column. Eluents consisted of 0.1% formic acid in water (A) and 0.1% formic in acetonitrile (B) and were used to generate a flow of 5% B for 5 min followed by a linear gradient to 90% B over 45 min at a flow rate of 4 μL/min. Data dependent analysis was conducted for all LC−MS/MS analyses by first acquiring an ESI mass spectrum (m/z 400−2000) followed by CID and ETD analysis undertaken on the two most abundant peaks. A normalized collision energy of 35% was used for all LC−CID-MS analyses.

Derivatization and Sample Preparation. All disulfide bonds in the peptides or proteins were first reduced by adding a 5-fold molar excess of dithiothreitol (DTT) to the peptide or protein (10 μL, 1 mM) in aqueous solution and incubating at 40 °C for 1 h in the dark (per manufacturer's recommended protocol for optimal efficiency). Immediately following reduction, the reduced peptide samples were split into three aliquots; one for iodoacetamide (IAM) alkylation, one for APTA derivatization, and one for DML modification. IAM-alkylation was performed by adding iodoacetamide (4 μL, 1 M) in ammonium bicarbonate buffer (pH ~8.0, 100 mM). This reaction was allowed to proceed at room temperature for 1 h in the dark, followed by the addition of excess DTT to quench the alkylation reaction. The resulting products are ones in which the reduced thiols are converted to *S*-carboxamidomethyl groups. APTA derivatization was achieved by reacting the reduced peptide solution with APTA (16 μL, 1 mg/mL in 50:50 ACN/100 mM ammonium bicarbonate buffer, pH 8.0) overnight at room temperature in the dark followed by freezing to terminate the reaction. The resulting products are fixed-charge trimethylammonium *S*-alkyl cysteine derivatives. DML modifications were initiated by reacting *N*,*N*-dimethyl-2-chloro-ethylamine (1 μL, 1 mM in 1 M HEPES buffer, pH 7.8) for 3 h at room temperature, producing carboxyamidomethyl cysteines. After incubation, all peptides were desalted using Thermo Fisher PepClean C_{18} spin columns and diluted for MS analysis as described above.

For the BSA protein sample, 100 μL of immobilized TPCK-treated trypsin beads in 100 mM ammonium bicarbonate (pH ~8.0) were used to digest 10 μmol of protein. The protein was enzymatically digested overnight at 37 °C after which the beads were removed, and the resulting tryptic digest was subjected to IAM-alkylation or APTA-derivatization using the procedures outlined above.

Protein Identification. Protein samples were identified using Sequest search software using the following parameters: 2 maximum miscleavages, ±0.5 Da fragment mass tolerance, ±1.0 Da precursor mass tolerance, and the appropriate fixed modification of cysteine (either the iodoacetamide alkylation or the APTA alkylation).

RESULTS AND DISCUSSION

ETD of Model Peptides. It has been previously shown that the addition of a fixed charge to a peptide can lead to a significantly different fragmentation pattern from its native form.[17,31−33] For example, fixed-charge derivatization schemes have been utilized extensively to simplify tandem mass spectra through the elimination of one series of fragment ions using a variety of different activation methods including PSD,[32] CID,[33] IRMPD,[34] ECD,[17] and recently in our group with electron transfer with gentle collision activation (ETcaD).[35] In the present study, we aim to improve the sequence coverage of peptides upon ETD analysis by increasing the charge states of peptides via attachment of quaternary amines to reduced cysteine residues. Cysteine residues are a logical choice for implementation of selective derivatization strategies owing to their high reactivity and low abundance in proteins which allows streamlined subsets of peptides to be targeted. As demonstrated in this study, the APTA derivatization procedure selectively and efficiently derivatizes all reduced cysteine residues and leads to higher charge states that result in greater sequence coverage, including production of key diagnostic ions that allow characterization of both the C- and N-termini. We also compare the ETD results obtained for APTA-derivatized peptides having fixed charge sites to peptides in which the cysteines are reduced and IAM-alkylated and to the same peptides for which the reduced cysteine residues are converted to dimethyl lysine analogues, which are more basic moieties that also afford higher charge states (but not fixed charge sites).[22] IAM is commonly used to prevent the recombination of disulfide bonds in reduced peptides by formation of *S*-carboxamidomethyl derivatives which are easily ionized by electrospray ionization (ESI) and matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). The DML derivatization procedure was shown previously to substantially increase both ETD efficiencies and sequence coverage obtained for cysteine-rich peptides.[22]

A series of cysteine-containing model peptides were derivatized using the three alkylating reagents, IAM, DML, and APTA (Scheme 1), and subsequently characterized by CID and ETD mass spectrometry. The APTA alkylation reaction is extremely selective for available cysteines through the alkylation of their thiol groups following the reduction of the disulfide bonds.[33,40] The reaction, as shown in Scheme 1, results in the addition of 170 Da and a permanent positive charge at each thiol. It is well-known that molecules with fixed charges often have higher ionization efficiencies and thus yield more intense ions, enhancing

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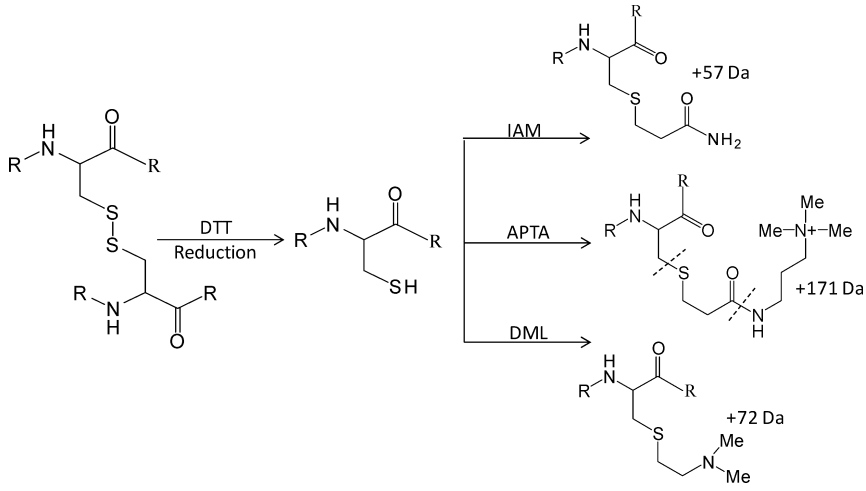
Scheme 1. DTT Reduction and Alkylation of Cysteines[a]



[a] The dotted lines represent cleavage sites upon ETD to form APTA reporter ions.

the detection of low-abundance cysteine-containing peptides in complex mixtures.[29,30,36] The APTA derivatization efficiencies are estimated to be approximately 70% based on the ratio of the summed areas of all APTA-derivatized products relative to the summed areas of all APTA-derivatized products and unmodified species detected in the full ESI mass spectra (data not shown).

Representative examples of the ETD mass spectra obtained for a cysteine-containing peptide, NRCSQGSCWN, after each of the three derivatization procedures (IAM, DML, and APTA) are shown in Figure 1. The highest charge state of the modified peptides were selected for ETD, corresponding to 2+ for the IAM-derivatized peptide, which has neither a fixed charge site nor an additional highly basic site; 3+ for the DML-derivatized peptide, which has an additional basic site; and 3+ for the APTA-derivatized peptide, which has a fixed charge site. As shown for the IAM-derivatized peptide in Figure 1A, very few sequence ions (c_6, c_7, and c_8) are observed upon ETD, providing incomplete sequence coverage (see inset in Figure 1A, which summarizes the sequence coverages for all three variations of the derivatized peptide). In addition, these three c-ions allow the position of only one cysteine residue to be pinpointed. For the DML-modified peptide (Figure 1B), the ETD mass spectrum displays far more complementary c and z sequence ions, and the resulting sequence coverage (see inset of Figure 1A) is greatly improved although there are still gaps near the termini as the c_2, c_9, z_1, and z_8 ions were not observed. The ETD mass spectrum for the APTA-derivatized peptide reveals the entire c series as well as nearly the entire z series of product ions (Figure 1C). The latter mass spectrum provides the most extensive sequence coverage and thus affords confident assignment of the peptide sequence, along with identification of the positions of both cysteine residues. Moreover, upon ETD the formation of fragment ions in multiple charge states is minimized for the APTA-modified peptides as illustrated in Figure 1C. Upon DML derivatization, a significant number of multiply charged product ions crowd the 500−700 m/z range which is consistent with previous results for ETD of DML analogues of other cysteine-containing peptides.[28]

A comparison of the ETD and CID results for a series of model peptides derivatized by the three methods is summarized in Figure 2. The number of sequence ions (c-, z-, and y-type ions for ETD and a-, b-, and y-type ions for CID) are displayed in bar graph form for the highest charge states of each modified peptide. The ETD efficiencies and sequence coverages were significantly diminished for the lower charged peptide cations as the dominant reaction pathway was charge reduction without dissociation (data not shown). For the ETD results, the APTA-modified peptides typically yielded the greatest number of c, z, and y ions. The sequence coverage of the APTA-modified peptides was consistently greater than that of the IAM-alkylated peptides and usually better than that obtained from the DML-modified peptides. For example, for bactenecin (RLCRIVVIRVCR), ETD of the triply charged IAM-alkylated peptide produced 1 y ion, 8 c ions, and 6 z ions, ETD of the DML-derivatized peptide (5+) yielded 1 y ion, 9 c ions, and 9 z ions, and ETD of the APTA-modified peptide (5+) produced 7 y ions, 11 c ions, and 11 z ions. Among the three types of derivatized peptides evaluated in the present study, the overall sequence coverage obtained upon ETD of the IAM-alkylated peptides was the least comprehensive as these derivatized peptides were predominantly observed in lower charge states due to the lack of additional highly basic or fixed charge sites. As previously noted, precursor cations in lower charge states tend to favor nondissociative processes upon electron attachment.[8,12,13]

Two contributing factors led to the observed enhancement in sequence coverage for the APTA-peptides relative to the DML-peptides. First, the APTA-modified peptides frequently undergo backbone cleavages close to the peptide termini upon ETD that were not routinely observed for the DML-peptides. These include cleavages between the terminal and penultimate residues leading to the formation of the single C-/N-terminal residue ion (e.g., c_1, z_1, and y_1) and their complementary fragment ions (e.g., z_{n-1}, c_{n-1}). Second, the production of the lower energy y fragment ion series upon ETD is enhanced for the APTA-peptides. The y fragment ions are commonly produced by CID, not ETD, but they are in fact reasonably abundant product ions observed upon ETD of the APTA-peptides. The increase in abundance of y ions for the APTA-peptides may be related to the presence of fixed charge sites at all cysteine residues. It has been proposed that the formation of y-type ions upon ECD is enhanced for

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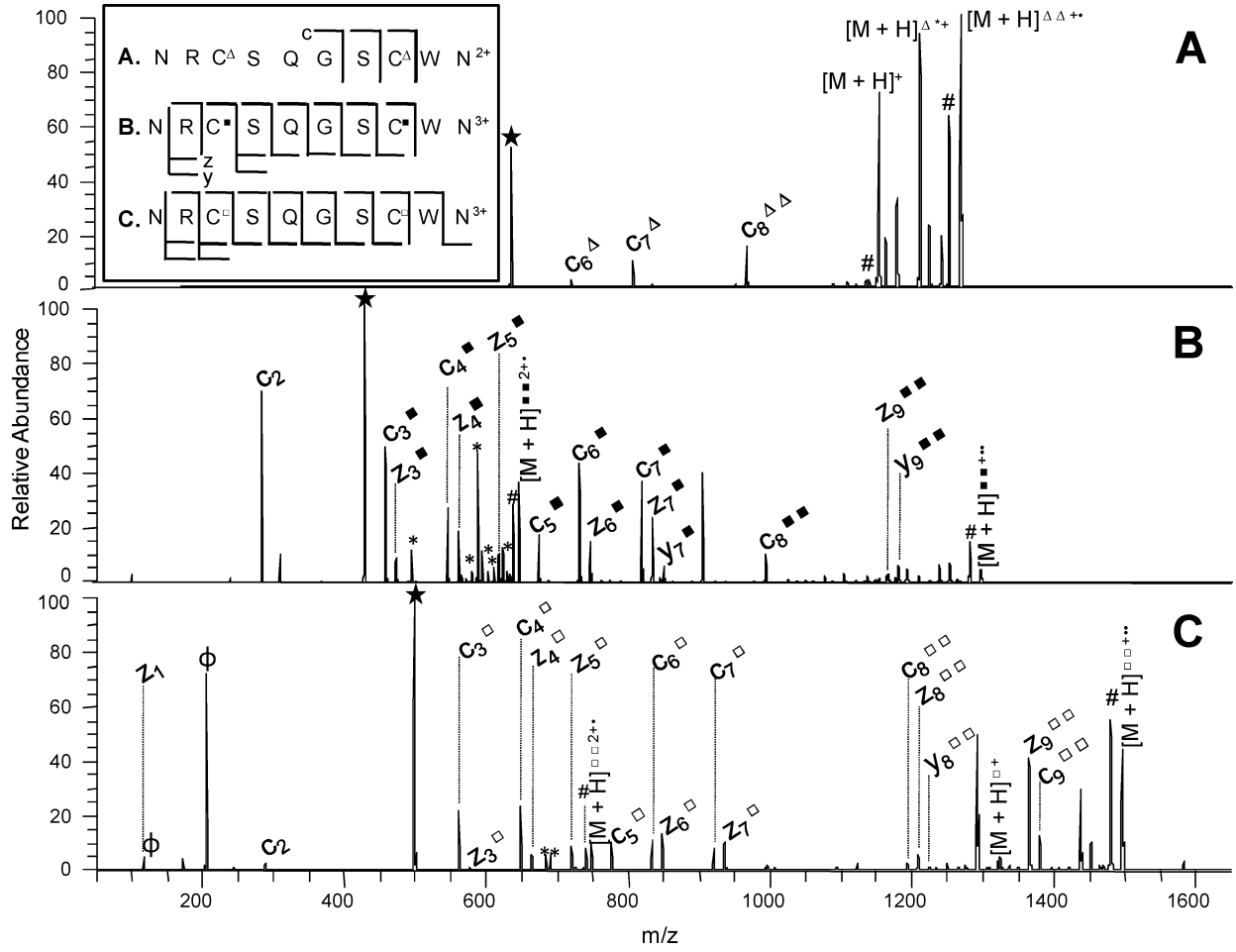

Figure 1. ETD product ion mass spectra of NRCSQGSCWN (A) doubly charged IAM-alkylated, (B) triply charged DML analogue, and (C) triply charged APTA-derivatized. IAM-alkylation is denoted by △, DML-modification by ■, APTA-derivatization by ○, APTA-peptide reporter ions by ϕ, multiply charged fragment ions by *, and neutral H_2O/NH_3 losses by #. The unmodified peptide is represented by M, while the selected precursor ion by ★.

peptides that can adopt zwitterionic structures as well as those peptides that have basic residues at the C-terminus or those with proline residues (i.e., peptides in which protons can migrate to the proline amide or other backbone amide positions).[37] Peptides that can adopt zwitterionic structures promote greater proton mobility, thus allowing proton localization at the backbone amide nitrogen.[36] The highly basic sites introduced at cysteine residues encourage the formation of zwitterions, presumably increasing the production of the y-type ions. Moreover, as ETD does not frequently yield products from the N-terminal side of proline residues,[7] there are often gaps in the sequence coverage. This shortcoming can be compensated (albeit at the expense of more elaborate data collection) by undertaking complementary CID analysis of the peptides because CID often causes enhanced cleavages N-terminal to proline residues.[38−40] However, the production of y ions stemming from cleavages N-terminal to proline residues was common upon ETD of the APTA-peptides in the present study, thus providing another set of N-terminal ions to improve the confidence in identifying the sequences. Both of these improvements can be visualized in Figure 2, in which the number of y ions produced is increased for the APTA-peptides in comparison to the DML-peptides and IAM-peptides.

Another way of assessing the analytical value of the ETD results for the IAM-, DML-, and APTA-derivatized peptides entails the comparison of the percent fragmentation values, defined by Coon et al. as the number of observed c- and z-type ions for a sequence divided by the total number of theoretically possible c- and z-type fragment ions.[41] Larger percent fragmentation values signify higher sequence coverage. The percent fragmentation is plotted as a function of the m/z values of the peptides in Figure 3. ETD of the APTA-peptides generally affords higher percent fragmentation values than that obtained for the IAM-peptides or DML-peptides. The decrease in percent fragmentation with increasing precursor m/z has been previously documented and leads to a precursor m/z limit for successful ETD.[41]

The ETD dissociation efficiencies, defined as the sum of the abundances of the z, c, and y ions divided by the total ion abundance (abundance of all ions including all backbone fragments, product ions due to neutral losses, reporter ions, charge reduced species and any surviving precursor ions), were calculated and expressed as percentages for the highest charge state (as listed in Figure 2) for the APTA-, DML-, and IAM-derivatized peptides. The ETD dissociation efficiencies for the APTA- and

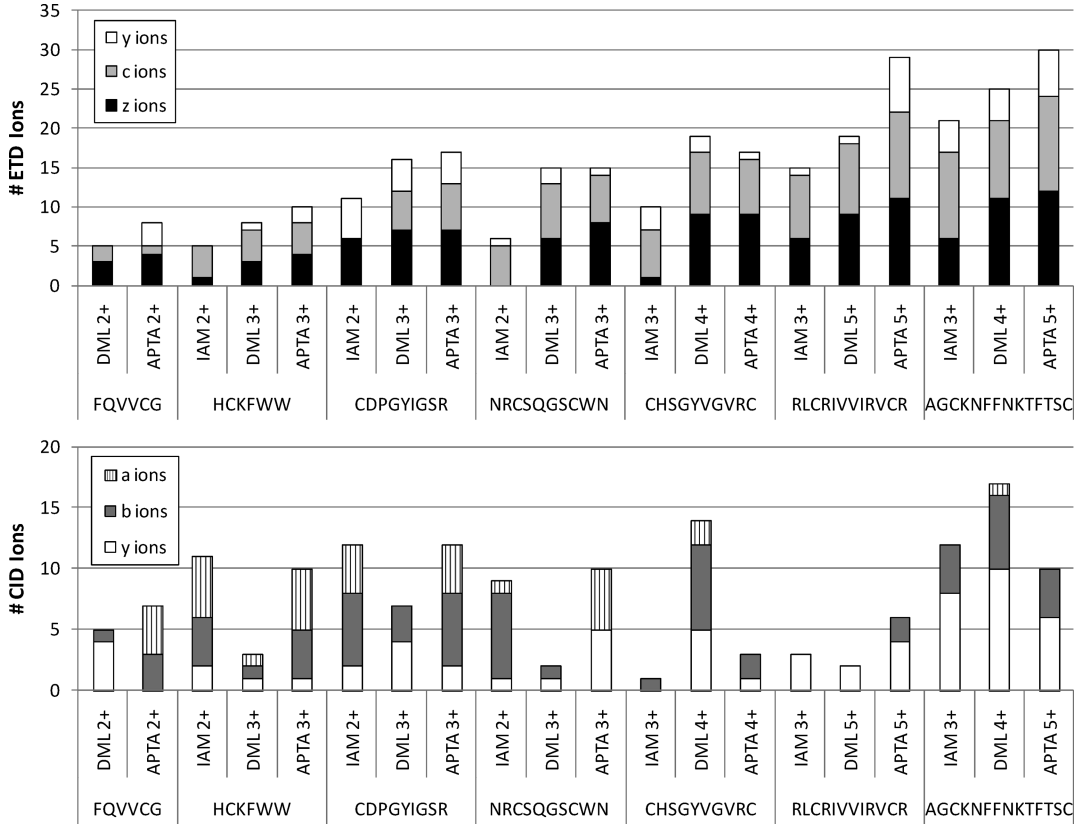

Figure 2. Number of diagnostic ions produced by ETD and CID for model peptides upon modification; IAM = IAM-alkylated, DML = dimethyl Lys analogue, APTA = APTA-derivatized.

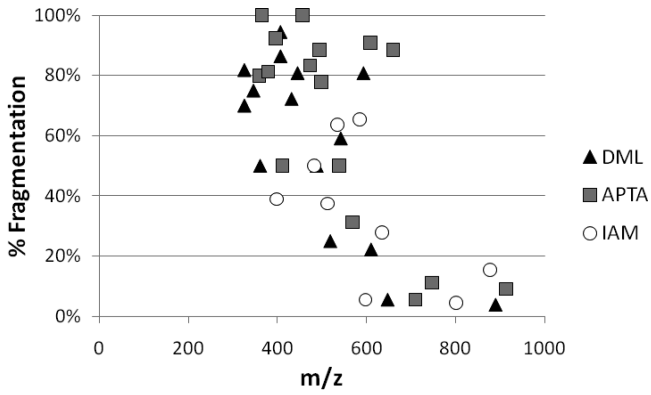

Figure 3. Percent fragmentation obtained upon ETD of model peptides as a function of precursor *m*/*z* and derivatization method. Percent fragmentation is defined as the number of observed fragment ions divided by the total number of possible fragment ions (all c- and z-type ions).

DML-peptides were comparable, averaging 47% and 53% for the series of model peptides, respectively, and only 18% for the IAM-peptides.

In addition to the benefits of the greater sequence coverage afforded upon ETD of the APTA-peptides, a group of characteristic reporter ions is also produced. Two reporter ions of *m*/*z* 117 and 205 offer a secondary means of readily pinpointing cysteine-containing peptides. These two reporter ions are likely produced through cleavage of the APTA moiety (*m*/*z* 117 and 205), as illustrated in Scheme 1 by the dashed cleavage lines. These ions are produced in reasonably high abundance for all charge states of the APTA-peptides, thus allowing consistent tracking of all of the APTA-peptides as seen in Figures 1 and 4. On the basis of the similar or better sequence coverage obtained for the peptides after APTA derivatization, the attachment of fixed charges (e.g., via APTA) rather than basic sites (e.g., via DML) was pursued for the remainder of the comparative study.

ETD of BSA Tryptic Digest. Following the assessment of the results obtained for the series of model peptides derivatized by the three reagents, tryptic peptides of bovine serum albumin were similarly analyzed. The BSA tryptic peptides were either alkylated with IAM or converted to APTA analogues for analysis by ETD and CID mass spectrometry. As anticipated, the IAM alkylation procedure, although efficient, led to the production of derivatized peptides in lower charge states (predominantly 2+ or 3+ depending on the length of the peptide) upon ESI than did the APTA derivatization (charge states of 3+ to 7+ observed). Consequently, ETD of the IAM-alkylated peptides largely resulted in nondissociative charge transfer yielding singly charged radical peptides and formation of few sequence ions. An example can be seen in Figure 4A for the tryptic peptide YNGVFQECCQAEDK, in which only seven c- and z-type ions and two y-type ions are observed, thus diminishing the identification of the peptide. In comparison,

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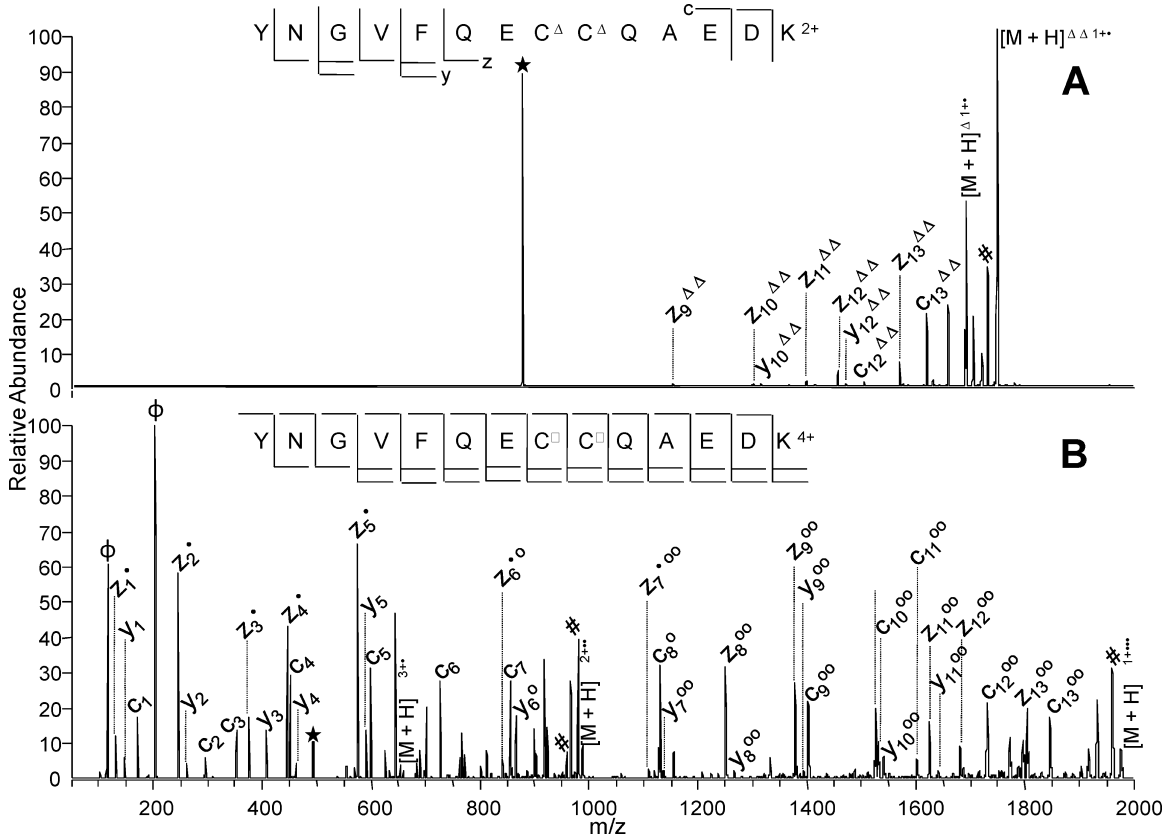

Figure 4. ETD product ion mass spectra of the BSA tryptic peptide YNGVFQECCQAEDK for the (A) doubly charged IAM-alkylated and the (B) quadruply charged APTA-derivatized. IAM-alkylation is denoted by △, APTA-derivatization by □, APTA-peptide reporter ions by ϕ, and neutral H_2O/NH_3 losses by #. The unmodified peptide is represented by M, while the precursor ion by ★.

ETD of the analogous APTA-modified peptide led to the production of far more sequence ions, including a complete series of c, z, and y ions and minor abundances of charge-reduced peptides (Figure 4B).

The results for the entire tryptic digest of BSA are summarized in Figure 5, which shows the number of diagnostic z, c, and all y and b ions upon ETD and y, b, and a ions upon CID for 25 IAM- and APTA-derivatized tryptic peptides. In order to maximize the number of diagnostic ions formed, ETD and CID were undertaken on the highest charge states observed for each of the IAM- and APTA-peptides. The APTA derivatization allowed analysis of many peptides that were either below the mass range or too low in abundance to be isolated upon conventional alkylation. For example, the two smallest peptides, CASIQK and GACLLPLK, were solely identified following the APTA derivatization/ETD strategy because without the APTA addition the m/z values of the multiply charged states of these small peptides fell below the m/z range analyzed. Moreover, the singly charged analogues with correspondingly higher m/z values do not produce charged products upon ETD. In addition, three peptides SLHTLFGELCK, SQYLQQCPFDEHVK, and AFSLQYLQQCPFDEHVKLVNELTEFAK were not detected after the IAM-alkylation procedure. In contrast, upon APTA derivatization these three peptides exhibited greater ionization efficiencies that allowed their detection and analysis in the data-dependent LC run.

There were three cases where the APTA-derivatized tryptic peptides were not detected (QNCDQFEK, ECCDKPLLEK, and CCAADDKEACFAVEGPK). Upon derivatization of all available cysteines and a corresponding increase in the charge states of these peptides, the m/z values of the resulting derivatized peptides fall below m/z 400 (the lowest mass scanned in the full mass spectra to eliminate LC solvent peak interferences) and thus these ions would not be detected. Despite this shortcoming, ~50% of BSA was characterized through the investigation of solely the APTA-derivatized cysteine-containing peptides. For all cases in which both IAM- and APTA-derivatized peptides were observed, ETD of the fixed charge APTA-peptides provided significantly greater sequence coverage than that obtained for the IAM-derivatized peptides. The same trend does not hold true for the analogous CID results. In some cases, the IAM-alkylated peptides yielded better sequence coverage, whereas in other cases the CID of the APTA-peptides gave greater sequence coverage. These CID results were not unexpected considering that the APTA-derivatization procedure adds fixed charges, not mobile protons that facilitate many of the fragmentation pathways observed by CID.[42,43]

As described earlier, a second way of assessing the analytical value of the ETD results for the derivatized peptides involves comparing the percent fragmentation values. The percent fragmentation values are plotted as a function of the m/z values of the derivatized tryptic peptides in Figure 6. ETD of the APTA-peptides generally affords a higher percent fragmentation values than that obtained for the IAM-peptides, again reflecting the merits

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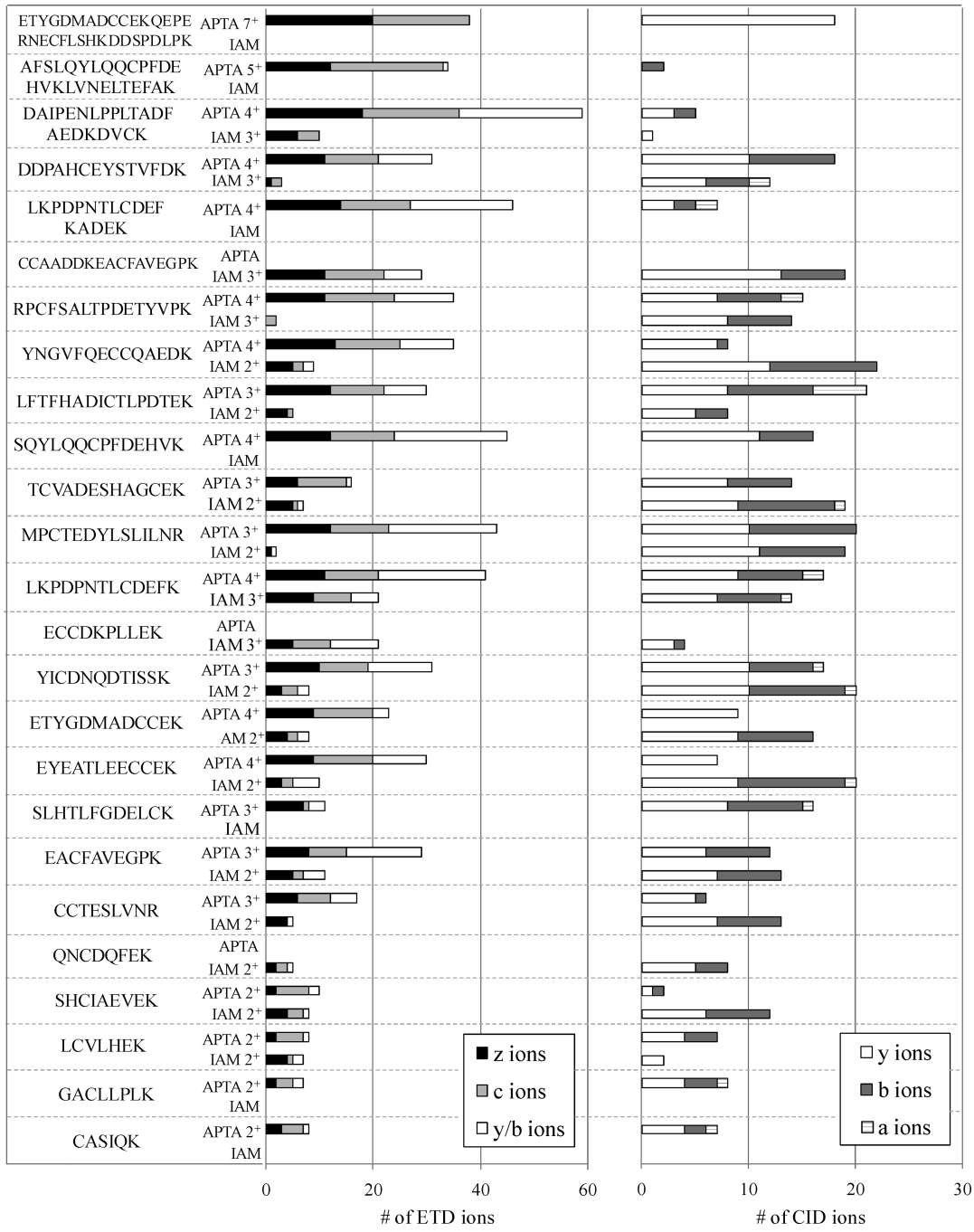

Figure 5. Number of diagnostic ions produced by ETD and CID for BSA tryptic peptides; IAM = IAM-alkylated, APTA = APTA-derivatized. If no bar is present, that derivatized peptide was not detected in any charge state and could not be analyzed.

of employing the fixed charge derivatization procedure for cysteine-containing tryptic peptides.

The ETD dissociation efficiencies were also calculated for the series of derivatized tryptic peptides shown in Figures 5 and 6. The dissociation efficiencies for the APTA-peptides averaged 56%, whereas the dissociation efficiencies for the IAM-peptides averaged only 17%. As expected from the well-recognized charge state dependence of ETD, the more highly charged APTA-peptides yield greater relative abundances of informative sequence ions upon ETD. This consistent increase in sequence ions also improves the application of search algorithms used to identify peptide sequences, thus exploiting the advantage of fixed charge derivatization in conjunction with ETD. As proof of concept, the HPLC−ETD-MS data obtained for each of the alkylated tryptic digests was submitted to a Sequest search procedure using the bovine taxonomy database. In both cases BSA was identified using the search parameters described in the experimental section. On the basis of the IAM-alkylated tryptic digest, a total of 21 peptides were correctly identified with high confidence, leading to a Sequest score of 582 and a sequence coverage of 55%. For the APTA-modified tryptic digest, the search was limited to only APTA-modified cysteine peptides, yielding the correct identification of 15 peptides with a score of 3700 and a 23% sequence coverage. This 6-fold increase in score even when accompanied by a decrease in sequence coverage proves that the selective study

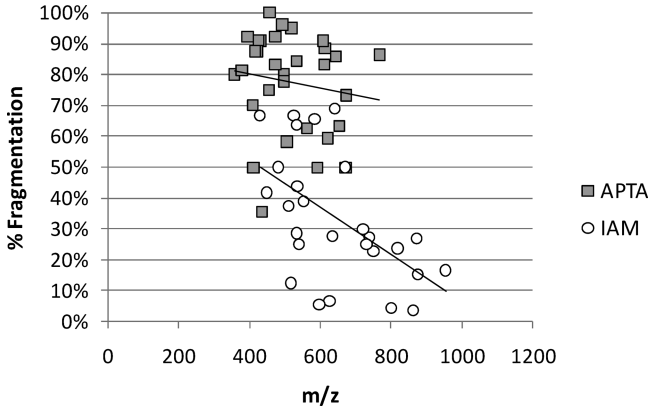

Figure 6. Percent fragmentation obtained upon ETD of tryptic peptides from BSA as a function of the precursor *m*/*z* and derivatization method. Percent fragmentation is defined as the number of observed fragment ions divided by the total number of possible fragment ions (all c- and z-type ions).

of tagged cysteine-containing peptides from a complex mixture affords a powerful identification tool in database searches.

CONCLUSIONS

The cysteine-selective fixed charge reagent, APTA, offers advantages over other charge enhancement strategies and traditional alkylation procedures in conjunction with ETD mass spectrometric analysis of peptides. The APTA derivatization procedures yields peptides in higher charge states upon ESI, and ETD of the APTA-modified peptides results in the formation of a nearly complete series of c, z, and y ions. The APTA modification increased the sequence coverage by enhancing fragmentation near the peptide termini as well as production of y ions not typically observed upon ETD. ETD of the APTA-peptides also resulted in higher fragmentation percentages (number of observed fragment ions/number of theoretically possible fragment ions) and greater dissociation efficiencies than obtained for conventional IAM-alkylated peptides. Generation of a pair of reporter ions upon ETD of the APTA-peptides offered a convenient means of tracking the cysteine-containing peptides.

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